

Please enter the following amendments and remarks.

AMENDMENTS

Please enter the following amendments:

In the specification:

Please replace the Title with the Title as shown below:

*Changed
in Title*

Anti-Angiogenic alpha-v-beta-3 Integrin-Binding Collagen
Peptides and Methods of Use Thereof

Please replace the Abstract with the Abstract as shown below:

b1

Anti-angiogenic proteins and peptides isolated from the non-Goodpasture region of $\alpha 3(\text{IV})$ NC1 domain of collagen are disclosed, which have the ability to bind $\alpha_v\beta_3$ integrin, and/or inhibit proliferation of endothelial cells.

Please replace the paragraph at page 20, lines 17 through 22, with the following paragraph:

b2

Figs. 24A, 24B, 24C and 24D are a set of four histograms showing binding of HUVEC cells to plates coated with Tumstatin (Fig. 24A), or controls of type IV collagen (Fig. 24B), vitronectin (Fig. 24C) or laminin-1 (Fig. 24A) in the presence of integrin

subunits α_1 through α_6 , β_1 , or $\alpha_v\beta_3$ integrin blocking antibody.

The plate coating is listed at the top of each graph, and the antibodies used for incubation are on the x-axis of each graph.

BSA-coated plates were used as negative controls.

Please replace the paragraph at page 43, line 23 through page 44, line 5, with the following paragraph:

One such fragment, designated "Tumstatin N-53", was found to have anti-angiogenic activity equivalent to that of full-length Tumstatin, as determined by standard assays. Tumstatin N-53 comprises a Tumstatin molecule wherein the N-terminal 53 amino acids have been deleted. Other mutant fragments described herein have been found to have very high levels of anti-angiogenic activity, as shown by the assays described herein. These fragments, "Tumstatin 333," "Tumstatin 334," "12 kDa Arresten fragment," "8 kDa Arresten fragment," and "10 kDa Canstatin fragment" have ED₅₀ values of 75 ng/ml, 20 ng/ml, 50 ng/ml, 50 ng/ml, and 80 ng/ml, respectively. By contrast, full-length Arresten, Canstatin and Tumstatin were found to have ED₅₀ values of 400 ng/ml, 400 ng/ml, and 550 ng/ml, respectively. Tumstatin 333 comprises amino acids 1 to 124 of SEQ ID NO:10, and Tumstatin 334 comprises amino acids 125 to 244 of SEQ ID NO:10.

Please replace the paragraph at page 47, lines 20 through 26 with the following paragraph:

B4
Identity is often measured using sequence analysis software *e.g.*, BLASTN or BLASTP (available at the world wide web site ("www") for the National Center for Biotechnology Information ("ncbi") of the National Institutes of Health ("nih") of the U.S. government (.gov"), in the "/BLAST/" directory). The default parameters for comparing two sequences (*e.g.*, "Blast"-ing two sequences against each other) by BLASTN (for nucleotide sequences) are reward for match = 1, penalty for mismatch = -2, open gap = 5, extension gap = 2. When using BLASTP for protein sequences, the default parameters are reward for match = 0, penalty for mismatch = 0, open gap = 11, and extension gap = 1.

Please replace the paragraph at page 105, line 2 through page 106, line 7 with the following paragraph:

b5
The nucleotide (SEQ ID NO:9) and amino acid (SEQ ID NO:10) sequences for the $\alpha 3$ chain of the NC1 domain of Type IV collagen are shown in Figs. 18A and 18B, respectively. The sequence encoding Tumstatin was amplified by PCR from the $\alpha 3$ NCI (IV)/pDS vector (Neilson, E.G. *et al.*, 1993, *J. Biol. Chem.* 268:8402-5; GenBank Accession Nos. M92993 (Quinones, S. *et al.*, 1994), M81379 (Turner, N. *et al.*, 1994), and X80031 (Leionin, A.K., and Mariyama, M. *et al.*, 1998)) using the forward primer 5'-CGG GAT CCG GGT TTG AAA GGA AAA CGT-3' (SEQ ID NO:11) and the reverse primer 5'- CCC AAG CTT TCA GTG

TCT TTT CTT CAT-3' (SEQ ID NO:12). The resulting cDNA fragment was digested with *Bam*HI and *Hind*III and ligated into predigested pET22b(+) (Novagen, Madison, Wisconsin, USA). The construct is shown in Fig. 19. The ligation placed Tumstatin downstream of and in-frame with the *pelB* leader sequence, allowing for periplasmic localization and expression of soluble protein. Additional vector sequence was added to the protein encoding amino acids MDIGINSD (SEQ ID NO:13). The 3' end of the sequence was ligated in-frame with the polyhistidine tag sequence. Additional vector sequence between the 3' end of the cDNA and the his-tag encoded the amino acids KLAAALE (SEQ ID NO:14). Positive clones were sequenced on both strands.

bs Plasmid constructs encoding Tumstatin were first transformed into *E. coli* HMS174 (Novagen, Madison, Wisconsin, USA) and then transformed into BL21 for expression (Novagen, Madison, Wisconsin, USA). Overnight bacterial culture was used to inoculate a 500 ml culture in LB medium (Fisher Scientific, Pittsburgh, Pennsylvania, USA). This culture was grown for approximately 4 hours until the cells reached an OD₆₀₀ of 0.6. Protein expression was then induced by addition of IPTG to a final concentration of 1 mM. After a 2-hour induction, cells were harvested by centrifugation at 5,000 x g and lysed by resuspension in 6 M guanidine, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0. Resuspended cells were sonicated briefly, and centrifuged at 12,000 x g for 30 minutes. The supernatant fraction was passed over a 5 ml Ni-NTA agarose column (Qiagen, Hilden, Germany) 4-6 times at a speed of 2 ml per minute. Non-specifically bound protein was removed by washing with both 10 mM and 25 mM

imidazole in 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0.

bs Tumstatin protein was eluted from the column with increasing concentrations of imidazole (50 mM, 125 mM, and 250 mM) in 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0. The eluted protein was dialyzed twice against PBS at 4°C. A portion of the total protein precipitated during dialysis. Dialyzed protein was collected and centrifuged at approximately 3,500 x g and separated into insoluble (pellet) and soluble (supernatant) fractions.

Amendments to the specification are indicated in the attached "Marked Up Version of Amendments" (pages i - v).

In the Claims

Please amend claims 4 and 9 as follows:

b6 4. (Twice Amended) An isolated fragment of α 3(IV) NC1 domain, having the amino acid sequence of amino acid residue 53 to amino acid 123 of SEQ ID NO:10.

b7 9. (Amended) An isolated fragment of α 3(IV) NC1 domain, having the amino acid sequence of amino acid residue 180 to amino acid residue 245 of SEQ ID NO:10.

Amendments to the claims are indicated in the attached "Marked Up Version of Amendments" (page vi).